CHAPTER 6

IN VITRO FERTILIZATION OF GOAT OOCYTES OBTAINED FROM IN VITRO MATURATION USING FROZEN-THAWED SEMEN AND SUBSEQUENT CULTURE OF EMBRYOS IN VITRO

6.1. SUMMARY

A study on in vitro fertilization of goat oocytes obtained from in vitro maturation using frozen-thawed semen and subsequent culture of embryos in vitro has been investigated. A total of 544 cumulus-oocyte complexes (COCs) were collected from 10 replicates. The oocytes were then incubated in TCM 199 medium at 39.0°C in 5%CO_2 and 95% air for 20 hr. Frozen goat semen was thawed at 37.0°C for 2 min and diluted with sperm washing medium and washed by centrifugation at 600xg for 5 min and subjected to swim-up procedure for 1 hr in the incubator. After cultured, 198 good quality of oocytes with expanded cumulus cells were chosen, the cumulus cells were removed, and the oocytes were divided into three groups: Group 1, the oocytes were inseminated with sperm in the fertilization medium supplemented with heparin (0.05 mg/ml), without oviductal epithelial cells; Group 2, the oocytes were transferred into fertilization medium supplemented with heparin and co-cultured with epithelial cells but were not inseminated; and in Group 3,

the oocytes were inseminated with sperm in the fertilization medium supplemented with heparin and also co-cultured with epithelial cells. At 16 hr post-insemination the oocytes/embryos were washed and transferred into in vitro culture (IVC) medium also added with epithelial cells. Cleavage of embryos was taken to indicate that fertilization has taken place. Based on this criterion, there was no fertilization in Groups 1 and 2 oocytes. In Group 3, 12.5% (7/56) of oocytes cleaved into 2-cell. Further culture of embryos have resulted on further cleavage in 5.3% (3/56) of them into 4-cell stage. The failure of oocytes co-cultured with epithelial cells but not inseminated to cleave suggests that the embryos obtained through the present system were unlikely as a result of parthenogenesis. The study also showed that the failure of oocytes to cleave in an epithelial cell-free system, suggests that the growth factor(s) that was possibly secreted by the epithelial cells is necessary for both fertilization and subsequent preimplantation embryonic development in vitro. The failure of embryos to further cleave beyond 4-cell stage implies that there is a developmental block between 4- to 8-cell stage.

6.2. INTRODUCTION

It has been shown that goat oocytes obtained from ovaries of slaughtered animals resume meiosis (Deb and

Goswami, 1990) and undergo ultrastructural cytoplasmic changes (Chapter 4) when cultured in vitro. In the final stage of second meiotic division, the oocytes reach metaphase II (MII) (Chapter 5). At that stage, the development of oocyte is arrested and the subsequent meiotic maturation occurs only when there is penetration by spermatozoon during fertilization (Nalbandov, 1976).

In vitro fertilization (IVF) with development to live young has been reported in several farm animals including cattle (Brackett *et al.*, 1982; 1984), sheep (Cheng *et al.*, 1986; Crozet *et al.*, 1987a), pig (Cheng *et al.*, 1986) and goats (Hanada, 1985; Jufen *et al.*, 1991). Ultrastructural studies of goat embryos obtained in vivo have also been reported (Crozet *et al.*, 1987b). Kim (1981) preincubated epididymal sperm for 5 to 10 hr in a m-Kreb medium or in isolated uterus from an oestrus sow and doe, and then cultured the sperm with in vitro matured zona-free goat oocytes. He found that 8 out of 10 oocytes were penetrated. Song and Iritani (1985) found that about 50% of cumulus-oocyte complexes (COCs) which had been collected from the ovaries of immature goat treated with gonadotrophin and cultured for 25 hr were penetrated by sperm. Younis *et al.* (1991, 1992) reported a successful IVF and pregnancy using non-superovulated and superovulated goat, however, in both studies the pregnancies did not continue to term.

The importance of co-culture system in the success rate of preimplantation embryonic development has been established (Gondolfi and Moor, 1987; Eyestone and First, 1989). In goat, attempt to undertake this procedure has been carried out by Sakkas *et al.* (1989) but in those studies the embryos were obtained through in vivo fertilization. Younis *et al.* (1992) have co-cultured the embryos with cumulus cells monolayers and found that 56% of ova developed to morulae from oocytes matured in the presence of LH than in FSH (41%), TSH (29%) or control (9%). Reports on the use of oviductal epithelial cells for co-culture of goat embryos obtained through IVF are lacking. The purpose of the present study was to perform IVF procedure using in vitro matured oocytes, and subsequently to develop such embryos in vitro with and without oviductal epithelial cell co-culture system.

In order to achieve the goal of this study, the oocytes obtained through IVM procedure were inseminated with heparin-treated frozen-thawed sperm, and the embryos thus obtained were subsequently further cultured for development.

6.3. MATERIALS AND METHODS

6.3.1. Collection and Classification of Oocytes

The animals used in this study were as described in Chapter 3. They were slaughtered in the local abattoir and the ovaries were taken as decribed in 3.3.1. They were rinsed and kept in phosphate-buffered saline (PBS) at 39.0°C and transported within 45 min to the Animal Biotechnology-Embryo Laboratory (ABEL) at UKM and treated as before (3.3.2). In this study, only the cumulus-oocyte complexes (COCs), i.e. those with two or more layers of cumulus cells were collected and used. The cumulus-free oocytes were ommitted from the study due to the inferiority of these oocytes based on the findings in the preceding chapters (Chapters 4 and 5).

A total of 544 COCs were collected from 10 replicates. After cultured in the IVM medium for 20 hr (Section 6.3.2), 198 good quality oocytes with expanded cumulus cells were chosen and divided into three Groups as follows:

Group 1:

One hundred and seven oocytes were inseminated in the fertilization medium and further cultured without epithelial cells.

Group 2:

Thirty five oocytes were co-cultured with epithelial cells but was not inseminated.

Group 3:

Fifty six oocytes were inseminated in the droplet of fertilization medium and co-cultured with epithelial cells.

6.3.2. In Vitro Maturation of Oocytes

The medium used for in vitro maturation (IVM) was modified TCM 199 described by Younis *et al.* (1991) (Section 3.4.3 of Chapter 3). The washed COCs were transferred into a 500 μL droplet of this medium and placed in a humidified 5% CO_2 in air at 39.0°C for 20 hr. At the end of maturation period, the oocytes with expanded cumulus and corona cells were collected in the hepes-buffered TCM 199 medium, and vortexed to remove the cumulus cells. Cumulus cell expansion has been taken to indicate that oocytes were developing and can be used for IVF. These oocytes were then washed four times with the same medium and rinsed once with the fertilization medium.

6.3.3. Preparation of Oviductal Epithelial Cells

Oviductal epithelial cell co-culture system were prepared as described in section 3.7 of Chapter 3.

6.3.4. Capacitation of Sperm and In Vitro Fertilization

Frozen semen of fertile local goats was used for IVF. The straw of semen was thawed in water bath at 37.0°C for 2 min. The semen was diluted ten-fold with sperm medium as previously described (section 3.4.4. of Chapter 3). The medium was then added with heparin 0.05 mg/ml (sodium salt, Sigma, Cat. No. H 5765). The sperms were washed two times by centrifugation at 600xg for 5 min. The sendimented spermatozoa were subjected to swim-up procedure using the same medium for 1 hr in the CO_2 incubator. The 200 μL droplets of heparin-treated fertilization medium was equilibrated for minimum 1 hr in the 5%CO_2 incubator at 39.0°C.

After equilibration, 20 μL of the top part of medium containing sperm was taken and inseminated into droplets containing oocytes. The concentration of sperm in the droplet of fertilization medium was between 1 to $2x10^6$/ml. 0.2 ml of the oviductal epithelial cells were added to the droplets. The droplets were then incubated at 39.0°C in 5%CO_2 and 95% air for 15 to 20 hr.

6.3.5. Evaluation of Fertilization and Embryonic Development In Vitro

Sixteen hour post-insemination, the oocytes were washed four times in hepes-buffered TCM 199 to remove the sperm. The oocytes were rinsed once with IVC medium and transferred into the 200 μL droplet of the same medium. 0.2 ml of oviductal epithelial cells were added to the droplets. The embryos were then incubated in the 5%CO_2 incubator and their development were observed every 24 hr.

6.4 RESULTS

Immediately after insemination, the sperms tried to penetrate the oocytes through the zona pellucida (Fig. 6.1). Occasionally, about 20 hr post-insemination, two polar bodies, indicating fertilization may have taken place could be observed (Fig. 6.2). In this study, however, the oocyte was said to be fertilized when there was a cleavage of minimum 2-cell (Fig. 6.3).

The rate of fertilization of goat oocytes with and without oviductal epithelial cells, and with and without sperm is summarised in Table 1. Cleavage did not take place among oocytes either without oviductal epithelial cells (Group 1) or among those without sperm (Group 2) and

the percentage of 2-cell embryos was 0.0% for both groups. For the oocytes inseminated as well as co-cultured with oviductal epithelial cells, 7 out of 56 (12.5%) oocytes cleaved into 2-cells (Fig. 6.3) and from these 7 embryos, 3 embryos (3/56; 5.3%) attained a 4-cell stage (Fig. 6.4).

In 2-cell embryo, normally one blastomere was bigger than the other (Fig. 6.3). When the two blastomeres divided into four blastomers, these new blastomeres were smaller than before (Fig. 6.4). The oocytes which did not cleave remain as a single cell (Fig. 6.5).

6.5. DISCUSSION

The aim of this study was to establish an IVF system whereby goat oocytes matured in vitro could be fertilized using frozen-thawed sperm and oviductal epithelial cells as co-culture, and the embryos produced were further cultured for cleavage. The previous report on the addition of oviductal epithelial cells in goat involved the use of in vivo matured oocytes (Sakkas *et al.*, 1989); Jufen *et al.*, 1991). The use of cumulus cells for co-culture in goat has also been undertaken (Younis *et al.*, 1992).

In vitro fertilization procedure requires methodologies such as sperm viability, sperm capacitation, oocyte maturation and fertilization. The present result, therefore, suggests that the above requirements may have been fulfilled such that fertilization has successfully taken place.

Successful fertilization obtained in the present study indicated that the frozen-thawed sperms were viable and fertilizable (Appendix 2). Such viability and fertilizability, also, indicated that the freezing technique used was sufficient (Appendix 1).

The present study also showed that 0.05 mg/ml heparin added to TCM 199 medium may have resulted in the capacitation of sperm. Younis *et al.* (1991) have noted that heparin appeared to be effective for in vitro capacitation of goat spermatozoa. Parrish *et al.* (1988) showed that glycoaminoglycans (GAG), including heparin, can induce acrosome reaction in bovine spermatozoa and improve both the frequency and quality of fertilization in vitro. Heparin also apparently binds to spermatozoa and plays a role in sperm uptake of calcium (Parrish *et al.*, 1988). Heparin has been shown to capacitate fresh or frozen-thawed bull sperm in a dose dependent manner that leads to fertilization rates above 70% (First and Parrish, 1988).

Besides heparin, other substances have been used to capacitate sperm. Hamster epididymal spermatozoa, for example, become capacitated after incubation for a few hours in a culture medium containing albumin (Yanagimachi and Chang, 1964; Bavister, 1973). Other methods include employing high ionic strength (Brackett *et al.*, 1982; Bondioli and Wright, 1983), bovine follicular fluid (Fukui *et al.*, 1983), caffein (Aoyagi *et al.*, 1988) and calcium ionophore (Byrd, 1981; Ben-Av *et al.*, 1988).

The 12.5% cleavage rate obtained in the present study appeared quite low, but comparison could not be made with other previous reports due to unavailability of such studies i.e. introduction of heparin-treated frozen-thawed sperms into the in vitro matured oocytes with oviductal epithelial cells as co-culture. It should be emphasized also that the criterion used for fertilization in this study was the cleavage of embryo. If the fertilization rate was determined on the basis of penetrated oocytes only, i.e. the presence of male and female pronunclei (Younis *et al.*, 1991) or sperm tail, such rate could have been higher. However the present study emphasized on the specific objectives and did not limit itself on the fertilization *per se*. It was the objective of this study to produce cleavage embryos which have a direct practical application in the future such as embryo transfer. Indeed, such practicality has been proven

by Hanada (1985) who transferred 2-cell embryos into recipient mothers and resulted in birth of kids. Beside this, experience on goats showed that it was very difficult to obtain oocytes due to the low slaughtering activity and the low number of usable oocytes per ovary. For these reasons, undue intervention on the fertilizing oocytes to examine the presence of pronuclei and sperm tail was avoided. Younis *et al.* (1991) demonstrated that when the in vitro matured oocytes, cultured in three different media, mDm, mTALP and mH-M199 were inseminated, the percentage of fertilized oocytes, indicated by the cleavage, were 33.3% (10/30), 10.0% (3/30) and 10.7% (3/28), respectively. The present finding of 12.5% fertilization rate using m-Kreb medium was therefore higher than that of Younis *et al.* (1991) using mTALP and mH-199.

The importance of oviductal epithelial co-culture system is obvious from this study. Out of 107 fully expanded oocytes (Group 1) inseminated, none of them showed any cleavage despite all other conditions being the same as in Group 3. The importance of these cells was not only for the development of early preimplantation embryos but also the fertilization itself. Comparison of this finding with that of others using the same species could not be made due to the scarcity of such studies. Sakkas *et al.* (1989) have used such cells in their study on goat preimplantation

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embryonic development but the oocytes were fertilized in vivo. However, studies on IVM-IVF involving co-culture with oviductal cells in bovine (Goto *et al.*, 1988; Eyestone and First, 1989; Iwasaki and Nakahara, 1990; Goto *et al.*, 1994), pigs (White *et al.*, 1989), sheep (Gondolfi and Moor, 1987), horses (Ball and Altschul, 1990) and human (Sathananthan *et al.*, 1990) all suggested that embryos produced were of better quality than those produced in cell-free culture.

Although the exact function of co-culture in the development of embryos was not known, it has been speculated that the somatic cells used for co-culture may produce unknown, embryo growth promoting factor(s) and/or the removal of embryo-toxic factors (s) from the culture medium. The former hypothesis is supported by the results of Heyman *et al.* (1987) who demonstrated the existence of a low molecular weight (<2700) peptide in embryotrophic fractions of medium conditioned by trophoblastic vesicles. Indirect support for the possibility that co-culture acted by removing inhibitory substances was from the demonstration of inhibitory compounds in the media commonly used for embryo culture. For example, Loutradis *et al.* (1987) found that hypoxanthine in Ham's F-10 medium induced a 2-cell block in mouse embryos, and pyruvate as a component of m-Kreb Ringer bicarbonate solution, was inhibitory to the development of early pig embryos (Davis and Day, 1978). Whether inhibitory

substances exist in the media used for goat embryo culture remains to be investigated.

There was always a problem when dealing with oviductal epithelial cells preparation, particularly as it is prone to contamination (Fig. 6.6). In the present study, it has been found that the epithelial cell preparation was prone to contamination by *Bacillus sp.* This does not take into account the difficulty in obtaining oviduct at the right stage of goat oestrus cycle. This problem arises because the goats normally slaughtered in the abattoir were few and of different reproductive status. In view of this problem probably in certain specific similar cases, an alternative co-culture system other than oviductal epithelial cells may be beneficial. In the bovine, for example, co-culture with trophoblastic vesicles (Heyman *et al.*, 1987), a trophoblastic cell strain (Scodras *et al.*, 1990), cumulus cells (Goto *et al.*, 1988; Chian and Niwa, 1994), embryonic chicken cells (Blakewood *et al.*, 1988), uterine cells (Voelkel *et al.*, 1985), fibroblast cells (Wiemer *et al.*, 1989) and transformed monkey-kidney cell line (VERO; Menezo *et al.*, 1990) have been established. Studies in goat using these cells are warranted.

The present study also showed that there was no embryo found in the sperm-free culture (Group 2). This finding

suggested that the cleaved embryos (Group 3) were the result of sperm penetration, and not the result of parthenogenesis. Parthenogenesis, the production of an embryo, with or without eventual development into an adult, from a female gamete in the absense of any contribution from a male gamete (Beatty, 1957), has been widely reported in laboratory animals such as mouse (Siracusa *et al.*, 1978; Kaufman, 1978; Cuthberston, 1983) and ferret (Chang 1957). Parthenogenesis in farm animals has also been reported such as bovine (Kono *et al.*, 1989; Nagai, 1992) and cattle (Nagai, 1987). In goat, such phenomenon remains to be investigated.

It was not possible from the present study to know whether there was any polyspermy among inseminated oocytes. This, again, was because the criterion of fertilization employed in the present study was cleavage, and not the presence of sperm tail in the ooplasm. Hanada (1985) found that 13 out of 34 in vivo matured goat follicular oocytes underwent polyspermy following IVF. Such a high rate of polyspermy could be partly due to the high concentration of sperm used (2.5×10^7/ml). In the present study the concentration of sperm used was 10 to 25 times lower (1 to 2×10^6/ml) than that of Hanada (1985) in which case the incidence of polyspermy, if occur, should had been lesser. At present, however, data on the optimum concentration of sperm to obtain an optimum rate of fertilization in goat is

lacking. Further studies on this aspect is therefore warranted.

The present study has shown that there was no further preimplantation embryonic development beyond 4-cell stage. Thus, it appears that there was a developmental block between 4- to 8-cell stage. Such a 4- to 8-cell block has also been reported in pig (Bavister, 1988). In their studies on embryonic development in goat, Sakkas *et al.* (1989) have found such a phenomenon at 8- to 16-cell stage , however, comparison could not be made with the present finding since in the former the embryos were obtain through in vivo fertilization. Nakayama *et al.* (1993) have noted that in hamster, the 2-cell block phenomenon was found in IVF but not in vivo. Preimplantation embryonic developmental block in a number of other mammalian species has also been previously reported. For example, in mouse the block is at the 2-cell (Goddard and Pratt, 1983), in sheep at 8- to 16-cell (Crosby *et al.*, 1988) and in bovine at 8- to 12-cell (Camous *et al.*, 1984) stage.

Although the mechanisms of the developmental block is still not clear, previous studies has shown that it is associated with the activation of embryonic genome (Goddard and Pratt, 1983; Bavister, 1988). It is also believed that the loss or decay of mRNA molecules of maternal origin,

developmental arrest in the presence of transcriptional inhibitors, and marked qualitative changes in protein synthetic patterns from one stage to the next become major features that characterize the timing of the switch from maternal to embryonic genome control (Telford *et al.*, 1990). Developmental block also might be related to oxygen toxicity, which results from exposure to high oxygen concentrations and light exposure during the manipulation of oocytes and embryos (Nakayama *et al.*, 1993).

The present IVF study indicated that the rate of cleavage of 12.5% was slightly better than that of Younis *et al.* (1991), however, the result of the present study and that of Younis *et al.* (1991) were lower than that of other livestock such as sheep (82.6%) (Cognie *et al.*, 1991) and pig (Yoshida, 1987). It should be emphasised, however, that the criteria of fertilization used by the latter two workers were penetration of the sperms and not cleavage, beside the fact that the animals used in the present study were of different ages and reproductive status.

Beside limitation, the present study, has nevertheless successfully established an in vitro fertilization procedure using oocytes matured in vitro and heparin-capacitated frozen-thawed sperm with oviductal epithelial cells as co-culture system.

Table 6.1. In vitro fertilization of goat oocytes with and without sperms and with and without oviductal epithelial cells

Groups of experiment	Number of oocytes with expanded cumulus cells	Epithelial cells		Sperm		2-cell (%)	4-cell (%)
		with (w)	without (w/o)	with (w)	without (w/o)		
1	107	-	w/o	w	-	0 (0.0)	0 (0.0)
2	35	w	-	-	w/o	0 (0.0)	(0.0)
3	56	w	-	w	-	7 (12.5)	3 (5.3)

Figure 6.1. A developing goat oocyte in which the cumulus cells have been removed and inseminated in the fertilization medium. Note the extrusion of the first polar body (Pb1). A number of sperms (s) tried to penetrate the zona pellucida (zp). cy, cytoplasm; ps, perivitellline space. Scale bar: 15 μm.

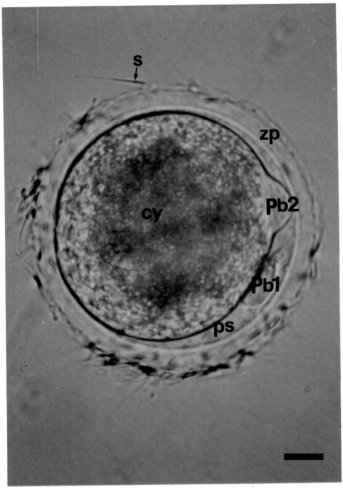

Figure 6.2 An embryo with extruded first polar body (Pb1) and extruding second polar body (Pb2) 20 hr post-insemination. cy, cytoplasm; zp, zona pellucida; ps, perivitelline space; s, sperm. Scale bar: 15 μm.

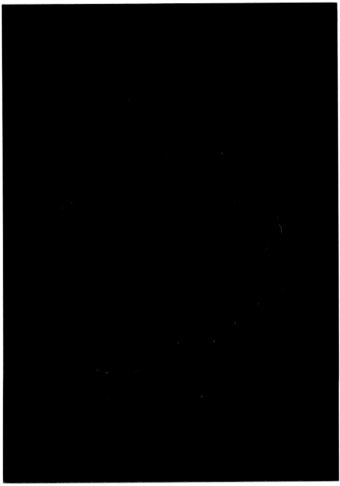

Figure 6.3. A 2-cell embryo, showing two blastomeres. The embryo was obtained through in vitro fertilization (IVF) procedure using oocytes matured in vitro (IVM) and subsequently cultured for 40 hr in vitro (IVC) co-cultured with oviductal epithelial cells. Note that one blastomere is bigger than the other. ps, perivitelline space. zp, zona pellucida. Scale bar: 15 μm.

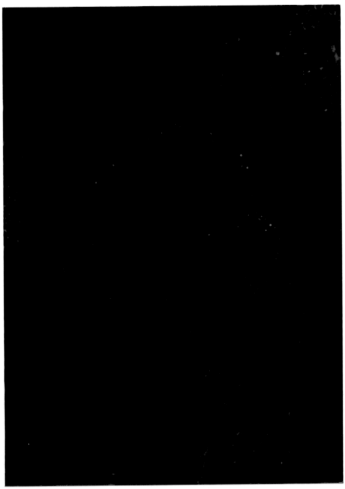

Figure 6.4. A 4-cell embryo of goat, showing four blastomeres. The embryo was obtained through in vitro fertilization (IVF) procedure using oocyte matured in vitro culture (IVC) co-cultured with oviductal epithelial cells (ec). zp, zona pellucida. Scale bar: 15 μm.

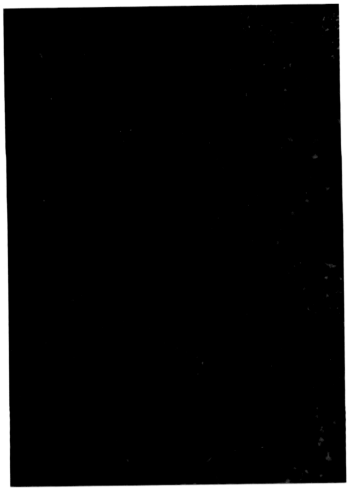

Figure 6.5. A single-cell goat oocyte/embryo 50 hr post-insemination and co-cultured with epithelial cells (ec). Note that the oocyte/embryo did not appear to cleave. cy, cytoplasm; zp, zona pellucida. Scale bar: 25 μm.

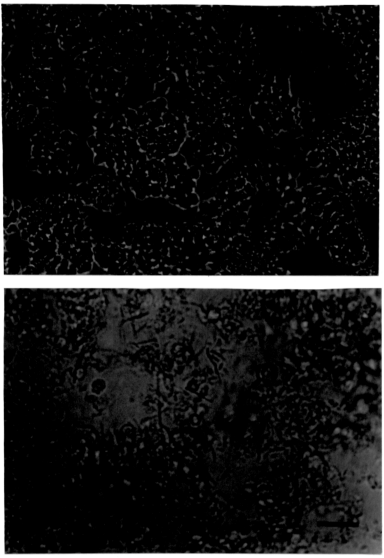

Figure 6.6. Oviductal epithelial cells (ec) taken from ampullary part of the oviduct during corpus hemorrhagicum stage. a) 6-days old, normal healthy; b) 4-days old, contaminated with *Bacillus sp.* Note the fragmented cells compared to those in (a). The yellow colour reflects the more acidic nature of the medium due to increase of CO_2 as a result of increase of metabolism by bacteria. Scale bar: a,b, 25 μm.